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(57) Abstract

A cell culture medium is provided which constrains cell growth and enhances antibody production. The high glucose medium of the invention is preferably saturated at 40 °C with essential amino acids.

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CELL CULTURE MEDIA FOR ENHANCED PROTEIN PRODUCTION

This application is a continuation of United States application Serial No. 08/833,500 filed 07 April 1997.

FIELD OF THE INVENTION

This invention relates to cell culture media, which improves protein production, constrains cell growth and extends cell longevity in vitro culture, and to methods for the production and use of such media.

BACKGROUND OF THE INVENTION

The increasing demand for monoclonal antibodies (MABs) useful in research, diagnosis, therapy and purification purposes has created a need to optimize production techniques. The prior art includes improved bioreactor designs and bioreactor operation to increase cell densities or the longevity of the culture by nutrient feedings.

Bioreactors have been operated in fed-batch, immobilized, perfusion and continuous modes. Alternate strategies, such as the use of temperature, media formulation, including the addition of mouse peritoneal factors, growth inhibitors, autocrine factors or cyclic mononucleotides and hyperstimulation by osmolarity stress, have been used to enhance protein production. These approaches have shown only marginal success.

Commonly used basal cell culture media are RPMI 1640, DMEM (Dulbecco's modified Eagle's medium), Ham's

F12 and DMEM/F12 (DF). Murakami (1989)(1)[1] describes a modified medium, eRDF, prepared from RDF(RPMI:DMEM: F12=2:1:1) by enrichment with amino acids, glucose and vitamins. Murakami showed that doubling total amino acids or glucose alone did not increase cell density but concurrent elevation of amino acids and glucose maximized the cellular growth by threefold. Hyper-stimulation of monoclonal antibody production by high osmolarity stress in a eRDF medium is described in Chua et al. (1994)(2) and (1994)(3). However, the maximum IgG concentration achieved was about 300 ug/ml and 270 ug/ml for HG11 and TBC3 cells, respectively, at medium osmolarities about 350 to 400 mOsm. Further increase in osmolarity with NaCl caused a deterioration in antibody production.

Oh, et al. (1995)(4) reports that hybridomas increased metabolic activities and amino acids uptake via the Na+ dependent symports to compensate for the osmotically elevated external environment.

Oh, et al. (1996)(5) describes the application of flow cytometry in examining the relationships between total cellular monoclonal antibody content, cell size, and cell cycle distribution of hybridomas subjected to environmental stress.

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[1] A bibliography precedes the claims.

SUMMARY OF THE INVENTION

The invention provides cell nutrient media which enhance protein production and prolong in vitro cell viability. The method cell culture utilizing such media are an important aspect of the invention.

The media and the methods of the invention are applicable to the culture of cells of any type in bioreactors of all kinds.

DETAILED DESCRIPTION OF THE DRAWINGS

Figure 1—growth of hybridomas 2HG11 and TBC3 in BTC-28101 and control DMEM/F12 media.

Figure 2a-2d reflect the result of hollow fiber bioreactor experiments in which BTC-28101 was utilized. Figure 2a represents levels of antibody produced. Figure 2b sets forth medium pH data. Figure 2c reports glucose utilization. Figure 2d reprots cell viability.

Figure 3a—growth of hybridoma 2HG11 in serum-free BTC-28101 and commercial media Hb and PFHM available from Gibco.

Figure 3b—IgG concentration in serum-free BTC-28101 and commercial media Hb and PFHM available from Gibco.

Figure 4—growth of hybridomas 2HG11 and TBC3 in BTC-28102 and control DMEM/F12 media.

Figure 5—growth of CHO cells in BTC-28103 and control IMDM media. IMDM refers to Iscove's Modified Dulbecco's Media.

Figure 6 presents a correlation of percent dry weight of amino acid in the media components with MAB production in ug/ml. The figure illustrates an unexpected increase in MAB concentration when the percent amino acid content in the media exceeds about 20%. In the Figure D/F refers to Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12, 1:1 Mixture.

DEFINITIONS

Cell Culture Medium—Any medium in which cells of any type may be cultured.

Bioreactor—Any device in which cells may be cultured. Includes stationary flasks, spinner flasks and hollow fiber bioreactors.

Basal Medium—A cell culture medium that contains all of the ingredients essential to cell metabolism, e.g., amino acids, lipids, carbohydrates, vitamins and mineral salts. RPMI, DMEM, Ham's 12 and RDF are examples of basal media.

Essential Amino Acids—Arg, Cys, Gln, His, Pro, Ile, Leu, Lys, Met, Phe, Thr, Trp, Tyr and Val.

Non-Essential Amino Acids—Ala, Asn, Asp, Gln, Gly, Ser.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides a method for improving protein production in cultures of protein producing cells. In particular, the invention comprises culturing hybridomas antibody producing cells in a high osmolarity

aqueous medium comprising a high concentration of amino acids, in particular the essential amino acids, and an energy source, such as glucose or sucrose. The medium is substantially saturated at around 40°C with an amino acid or acids essential to the metabolism of the culture cells. The medium of the invention contains 5.50 to 20 grams per liter of total or gross amino acids in solution or suspension and 5.50 to 20 grams per liter of a carbohydrate energy source, preferably glucose, in solution. The gross amino acids comprise at least 20%, preferably, from about 25% to about 50% of the total dry weight of the medium components. Cells may appropriately be adapted to the high osmolarity media of this invention by passaging.

The osmolarity of the medium is from 320 to 450. Sodium chloride is the preferred osmolyte.

The media and the methods of the invention are useful in all forms of bioreactors. The benefits of the invention are realized in static, batch, shaker flask, and spinner and hollow fiber bioreactor culture procedures.

Cells of all kinds may be cultured in any of the methods of the invention. Culture of recombinant protein expression mammalian cells, e.g., CHO cells, is an important aspect of the invention. Many types of mammalian cells, which contain recombinant protein containing expression vectors are known. See, e.g.,

Acklin, C., et al. (Recombinant human brain-derived neurotrophic factor (rHuBHDNF). Disulfide structure and characterization of BDNF expressed in CHO cells) Int.J.Pept.Protein Res. (1993) 41:548-52; Fukushima, K., et al., (N-linked sugar chain structure of recombinant human lymphotoxin produced by CHO cells; the functional role of carbohydrate as to its lectin-like character and clearance velocity) ABB (1993) 304:144-53; Hayakawa, T., et al. (In vivo biological activities of recombinant human erythropoietin analogs produced by CHO cells, BHK cells and C127 cells) Biologicals (1992) 20:253-7; Israel, D.I., et al. (Expression and characterization of bone morphogenetic protein-2 in Chinese hamster ovary cells) GF (1992) 7:139-50; Langley, K.E., et al. (Purification and characterization of soluble forms of human and rat stem cell factor recombinantly expressed by Escherichia coli and by Chinese hamster ovary cells) ABB (1992) 295:21-8; Lu, H.S., et al. (Post-translational processing of membrane-associated recombinant human stem cell factor expressed in Chinese hamster ovary cells) 298:150-8; Malik, N., et al., (Amplification and expression of heterologous oncostatin M in Chinese hamster ovary cells) DNA Cell Biol. (1992) 11:453-9; Nagao, M., et al. (Production and ligand-binding characteristics of the soluble form of murine erythropoietin receptor) Biochem. Biophys. Res. Commun. (1992) 188:888-97; Rice, K.G., et al. (Quantitative

mapping of the n-linked sialyloligosaccharides of recombinant erythropoietin; combination of direct high-performance anion-exchange chromatography and 2-aminopyridine derivatization) Anal. Biochem. (1992) 206:278-87; Schmelzer, C.H., et al. (Purification and partial characterization of recombinant human differentiation-stimulating factor) Protein Expr. Purif. (1990) 1:54-62; Schmelzer, C.H., et al. (Biochemical characterization of human nerve growth factor) J. Neurochem. (1992) 59:1675-83; Sima, N., et al. (Tumor cytotoxic factor/hepatocyte growth factor from human fibroblasts; cloning of its cDNA, purification and characterization of recombinant protein) Biochem. Biophys. Res. Commun. (1992) 180:1151-8; Sun, X.J., et al. (Expression and function of IRS-1 in insulin signal transmission) J. Biol. Chem. (1992) 267:22662-72; Suzuki, A., et al. (Biochemical properties of amphibian bone morphogenetic protein-4 expressed in CHO cells) BJ (1993) 291:413-7; Tressel, T.J., et al. (Purification and characterization of human recombinant insulin-like growth factor binding protein 3 expressed in Chinese hamster ovary cells) Biochem. Biophys. Res. Commun. (1991) 178:625-33. See also Lucas, B.K., et al. (High-level production of recombinant proteins in CHO cells using a dicistronic DHFR intron expression vector) (1996) Nucleic Acids Res. 24:1774-9.

EXAMPLE 1

Medium BTC 28101

The dry powder form of Medium BTC-28101 was prepared as two separate components (A) and (B) as listed in Table I. The ingredients were milled to fine dry powder prior to use. To prepare the medium, Component (A) was dissolved in 90% by volume of pyrogen-free water. The mixture was warmed to around 40°C and stirred for one hour to fully dissolve the powder, and then cooled down to room temperature. Component (B) was added and stirred another hour to dissolve. pH was adjusted to 7.0 by addition of NaOH. Water was added to make up to the desired volume. The osmolarity of the medium was in the range of 330-335 mOsm/Kg.

Table I - Composition of Medium BTC-28101 in mg/L

Component (A)	
Amino Acids	
Alanine	13.4
Arginine.HCl	1,162.9
Asparagine.H2O	189.2
Aspartic acid	80.0
Cystine.2HCl	105.4
Cysteine.HCl.H2O	105.4
Glutamic acid	79.4
Glutamine	1,997.2
Glycine	85.6
Histidine.HCl.H2O	150.9
Hydroxyproline	63.0
Isoleucine	314.8
Leucine	330.6
Lysine.HCl	394.6
Methionine	98.4
Phenylalanine	148.6

Proline	110.6
Serine	170.2
Threonine	221.6
Tryptophan	36.8
Tyrosine	174.0
Valine	218.0

Component (B)

Mineral Salts	
CaCl2 (anh)	82.1
CuSO4.5H2O	0.00075
FeSO4.7H2O	0.220
KCl	372.8
MgSO4 (anh.)	52.4
NaCl	6,136.2
Na2HPO4 (anh.)	484.1
ZnSO4.7H2O	0.230

Vitamins	
Biotin	0.102
D-Ca pantothenate	1.240
Folic acid	8.800
Putrescine.2HCl	0.040
Niacinamide	1.510
Para-aminobenzoic acid	0.510
Pyridoxine.HCl	0.520
Pyridoxal.HCl	1.000
Riboflavin	0.210
Thiamine.HCl	1.585
Vitamin B12	0.342

Carbohydrates and derivatives	
D-glucose	6,846.0
Na Pyruvate	110.0

Nucleic acid derivatives	
Thymidine	5.7
Hypoxanthine	1.0

Lipids and derivatives	
Choline bitartrate	55.7
i-inositol	104.5
Linoleic acid	0.020
Lipoic acid	0.050

Thiol compound	
Glutathione (reduced)	0.490

Buffers	
HEPES	3,570.0
NaHCO3	1,130.0

pH indicator	
Phenol red	6.0

The composition of the medium BTC-28101 is:

Glucose (mg/l)	6.846
Amino acids (mg/l)	6.251
Amino acids (%d.w.*)	24.8

*dry weight of media ingredients

EXAMPLE 2

Effect of Medium BTC-28101 on IgG Production in Hybridomas in Shaker Flask Culture

This example compares cell growth and monoclonal antibody production in two hybridoma cell lines 2HG11 (antihuman chorionic gonadotropin) and TBC3 (antihuman IgG) in the serum supplemented in the BTC-28101 medium of Example 1 versus DMEM/F12 (Dulbecco's modified Eagle's medium: Ham's F12=1:1).

The experiment was set up in shaker flasks with 100 ml media supplemented with 10% FBS (Fetal Bovine Serum). Inoculum cells were adapted and maintained by daily passaging at $2x10^5$/ml with the respective fresh medium for at least a week, and the viability of each inoculum culture was above 90% before use. Batch culture was started by inoculating at $2x10^5$/ml into the respective medium. Samples were taken daily to follow the cell growth by trypan blue staining and hemocytometer counting. Monoclonal antibody concentration in the culture supernatant was determined by ELISA analysis. The effect on cell growth is shown in Fig. 1. Maximum concentration of Ig at the end of the cultures are summarized in Table II:

Table II. Maximum Ig Concentration in the Cultures with BTC-28101 and Control DMEM/F12 Media

Cell Line	Max Ig Concentration (ug/ml)	
	DMEM/F12	BTC-28101
2HG11	50	270
TBC3	84	450

EXAMPLE 3

Hybridoma cell line TH12 (anti-theophylline) was cultured in either the BTC-28101 media of Example 1 or a DMEM formulation. Cells were inoculated into 100 ml of BTC-28101 or control medium DMEM at $2X10^5$/ml in 250 ml spinner flasks, both media were supplemented with 10% FBS. Similar procedure as stated in Example 2 was followed for preparing the inoculum cultures, and for monitoring the batch. TH12 produced higher concentrations of antibody in BTC-28101 than in the formulation of DMEM. As Table III shows, cell numbers and cell viability were also higher in 28101.

Table III

TH12 Batch Culture:Cell Counts and Viabilities

DATE	MEDIUM	CELL COUNT	VIABILITY
DO 1/30/95	BTC-28101 DMEM	2 x 10^5/ml 2 x 10^5/ml	100% 90%
D● 1/3●/95	BTC-28101 DMEM	5.6 x 10^5/ml 2.6 x 10^5/ml	100% 90%
D2 2/1/95	BTC-28101 DMEM	2.4 x 10^6/ml 0.8 x 10^6/ml	98% 91%
D3 2/2/95	BTC-28101 DMEM	3 x 10^6/ml 2.2 x 10^6/ml	98% 97%
D4 2/3/95	BTC-28101 DMEM	3.4 x 10^6/ml 1.4 x 10^6/ml	93% 77%
D5 2/4/95	BTC-28101 DMEM	3.8 x 10^6/ml 0.5 x 10^6/ml	69% 32%
D6 2/5/95	BTC-28101 DMEM	8.4 x 10^5/ml 3.6 x 10^5/ml	25% 20%
D7 2/6/95	BTC-28101 DMEM		5% day 7 only

Viabilities in both media were <5%.
Total media volumes collected for analyses.

Table IV demonstrates enhanced Ig production and specific antibody titer when BTC-28101 is used.

Table IV

TH12 Batch Culture: Ig Concentrations and Specific Antibody Titers.

DATE	MEDIUM	Ig mg/Ml[1]	TITER[2]
D1 1/31/95	BTC-28101 DMEM	539 ug/ml 447 ug/ml	1,600 400
D2 2/1/95	BTC-28101 DMEM	468 ug/ml 397 ug/ml	12,800 3,200
D3 2/2/95	BTC-28101 DMEM	681 ug/ml 440 ug/ml	12,800 6,400
D4 2/3/95	BTC-28101 DMEM	752 ug/ml 518 ug/ml	6,400 3,200
D5 2/4/95	BTC-28101 DMEM	823 ug/ml 553 ug/ml	25,600 6,400
D6 2/5/95	BTC-28101 DMEM	1,500 ug/ml 489 ug/ml	25,600 6,400
D7 2/6/95	BTC-28101 DMEM	1,190 ug/ml 560 ug/ml	25,600 3,200

[1] Ig concentrations determined by precipitating each sample with saturated ammonium sulfate and reading optical densities at 280 nm.

[2] Specific antibody titers determined in an indirect ELISA with theophylline-BSA on the solid phase.

EXAMPLE 4

Hybridoma cell line DI16 (anti-*Dirofilaria immitis*) was cultured in either the BTC-28101 or DMEM. DI16

produced higher concentrations of antibody in BTC-28101 than in the in-house formulation of DMEM.

Table V shows that cell numbers and cell viability were also higher in BTC-28101.

Table V

DI16 Batch Culture:Cell Counts and Viabilities

DATE	MEDIUM	CELL COUNT	VIABILITY
DO 3/31/95	BTC-28101 DMEM	2 x 10^5/ml 2 x 10^5/ml	98% 98%
D3 4/03/95	BTC-28101 DMEM	7.2 x 10^5/ml 8.4 x 10^5/ml	83% 99%
D4 4/04/95	BTC-28101 DMEM	5.4 x 10^5/ml 1.5 x 10^5/ml	57% 26%
D5 4/05/95	BTC-28101 DMEM	6 x 10^5/ml 0.5 x 10^5/ml	58% 8%
D6 4/06/95	BTC-28101 DMEM	3.9 x 10^5/ml -	30% 0%
D7 4/07/95	BTC-28101 DMEM	3.5 x 10^5/ml -	21% 0%
D8 4/08/95	BTC-28101 DMEM	1.8 x 10^5/ml -	10% 0%

Table VI reports comparative Ig titers and concentration.

Table VI

DI16 Batch Culture: Ig Titers and Concentrations

DATE	MEDIUM	Ig TITER[1]	Ig mg/ml[2]
D3 4/03/95	BTC-28101 DMEM	1:1,024 1: 256	1.09 0.770
D4 4/04/95	BTC-28101 DMEM	1:2,048 1: 512	0.882 0.926
D5 4/05/95	BTC-28101 DMEM	1:2,048 1: 512	0.940 0.654
D6 4/06/95	BTC-28101 DMEM	1:2,048 1: 512	1.28 0.746
D7 4/07/95	BTC-28101 DMEM	1:2,048 Culture terminated	1.11 Culture terminated
D7 4/07/95	BTC-28101 DMEM	1:2,048 Culture terminated	1.39 Culture terminated

[1] Ig titers determined by titrating samples in a mouse Ig capture ELISA.

[2] Ig concentrations determined by precipitating each sample with saturated ammonium sulfate and reading optical densities at 280 nm; mg/ml=O.D. 280 x dilution factor ÷ 1.41 extinction coefficient.

EXAMPLE 5

Hybridoma cell line NP11 (anti-N-acetylprocainamide) was cultured in either BTC-28101 or DMEM. This cell line was slightly slower than the other cell lines to respond to BTC-28101 with enhanced levels of antibody production;

variations for different cell lines are not surprising. It is significant that the BTC-28101 culture produced substantial levels of antibody when cultures in DMEM were no longer viable. The ability to keep cultures producing for longer periods of time is a significant advantage of BTC-28101. See Table VII.

Table VII

NP11 Batch Culture:Cell Counts and Viabilities

DATE	MEDIUM	CELL COUNT	VIABILITY
DO 4/07/95	BTC-28101 DMEM	2 x 10^5/ml 2 x 10^5/ml	100% 100%
D1 4/08/95	BTC-28101 DMEM	1.4 x 10^5/ml 2.3 x 10^5/ml	100% 100%
D3 4/10/95	BTC-28101 DMEM	8 x 10^5/ml 1.04 x 10^6/ml	98% 84%
D4 4/11/95	BTC-28101 DMEM	8.4 x 10^5/ml 6.7 x 10^5/ml	81% 55%
D5 4/12/95	BTC-28101 DMEM	1.2 x 10^6/ml 2.9 x 10^5/ml	72% 21%
D6 4/13/95	BTC-28101 DMEM	8.4 x 10^5/ml 1 x 10^5/ml	52% 0%
D7 4/14/95	BTC-28101 DMEM	7.6 x 10^5/ml -	41% 0%
D10 4/17/95	BTC-28101 DMEM	1.3 x 10^5/ml -	5% 0%

EXAMPLE 6

Effect of BTC-28101 on IgG Production in Hollow Fiber Culture

Performance of the hybridoma in a "mini" hollow fiber bioreactor (UniSyn Technologies, Inc.'s "Mini Mouse" bioreactor) supplied with FBS supplemented BTC-28101 was compared with the control FBS supplemented DMEM. The results are shown in Table VIII. Comparable levels of antibody were produced by this hybridoma in the control DMEM and in BTC-28101. However, the control culture was terminated after day 13 when viability was <10%. In contrary, the cells in BTC-28101 remained highly viable, and the culture was terminated only because of shortage of medium supply.

Table VIII. Comparison of Ig Titer of Hollow Fiber Culture in BTC-28101 and Control DMEM Media

Time (day)	BTC-28101	DMEM
2	1:6,400	1:51,200
5	1:102,400	1:204,800
7	1:204,800	1:204,800
9	1:204,800	1:102,400
12	1:204,800	1:102,400
14	1:204,800	-
16	1:102,400	-
19	1:51,200	-

EXAMPLE 7

Hollow Fiber Bioreactors—28101 Medium

This hollow fiber bioreactor experiment involved a particular cell line which normally produces a few hundred ug/ml of MAB in conventional method. Fig. 2a indicates that this cell line performed substantially better in BTC-28101. Data for pH of the medium (Fig. 2b), glucose utilization (Fig. 2c), and cell viability (Fig. 2d) are presented. Cells growing in BTC-28101 in hollow fiber bioreactors do not appear to utilize glucose from the medium at the rate normally seen with conventional media. Monitoring of glucose utilization is a standard means of monitoring the progress of cells in hollow fiber bioreactors—the higher the level of glucose utilization, the better the cells are growing.

EXAMPLE 8

Spinner Flask Experiments with BTC 28101 Medium

Anti-theophylline hybridoma cells were inoculated into 250 ml of Difco's preparation of BTC 28101 or DMEM at 2 x 10^5 cells/ml in 500 ml spinner flasks. Both media were supplemented with 10% FBS, 2% L-glutamine, and 1% pen-strep. Five ml samples were collected from each flask on the days indicated. Cell viability was determined each day samples were collected, and antibody concentrations were determined for all samples by radial

immunodiffusion (RID) after all had been collected. Until that time, the samples (with cell material removed by centrifugation) were stored at -20°C. Antibody concentrations, as determined by RID, and cell viabilities are described in Table IX:

Table IX

Day 0 = 12/30/96

	ug/ml Ab		Cell Viability	
Day	DMEM	Difco	DMEM	Difco
0	<125*	<125	95%	95%
1	<125	<125	94%	95%
3	<125	<125	95%	95%
4	<125	<125	58%	86%
7	<125	176	0	27%
8		562	0	21%
9		473	0	23%
14		1,035	0	19%

*The lowest concentration RID standard used was 125 ug/ml.

It is significant that the cell line utilized produced 1 mg/ml under conditions described. Specifically, the spinner flasks did not provide ideal culture conditions. Once the experiment was set up, the medium was never replaced or replenished. Consequently, metabolites and dead cells continued to accumulate.

EXAMPLE 9

Anti-theophylline hybridoma cells were inoculated into 100 ml of Difco's preparation of BTC 28101 or in-house medium (DMEM) at 2 x 10^5 cells/ml in 250 ml spinner flasks. All other parameters were as described in

Example 8. Antibody concentrations, as determined by RID, and cell viabilities are described in Table X:

Table X

Day 0 = 1/18/97

	ug/ml Ab		Cell Viability	
Day	DMEM	Difco	DMEM	Difco
1	<125*	<125	95%	99%
3	<125	156	87%	99%
4	<125	209	49%	74%
5	<125	436	16%	65%
6	<125	417	9%	39%
7	<125	417	0	14%
9		400	0	10%

*The lowest concentration RID standard used was 125 ug/ml.

Note that the culture volume in Example 9 was one-half that in Example 8. Consequently, nutrients may have depleted more quickly and metabolites or other materials accumulated in inhibitory concentrations more rapidly.

EXAMPLE 10

BTC-28101 on IgG Production in Serum-Free Culture

This example compares cell growth and monoclonal antibody production in a hybridoma cell line (2HG11) in serum-free BTC-28101 and other commercially available serum-free media available from Gibco.

Hybridoma 2HG11 has been adapted to serum-free conditions in the respective media. All media were supplemented with insulin, transferrin, ethanolamine and selenite. Cells were inoculated into 100 ml of BTC-28101

or control media at $2x10^5$/ml in 250 ml shaker flasks. The results on growth and IgG production are shown in Figs. 3a and 3b.

EXAMPLE 11

Preparation and Use of BTC-28102 to Culture Hybridomas

Nutrient contents of the Medium BTC-28101 was further enhanced to formulate Medium-28102. To prepare this medium, Component (C) was prepared according to the composition in Table XI and milled to dry fine powder. The powder was sterilized by gamma-irradiation and added to 100 ml of BTC-28101, constituting the Medium BTC-28102. Osmolarity of the medium was around 400 mOsm/Kg.

Table XI: Composition of Supplement to Medium BTC-28101 to Make Up BTC-28102

Component (C) in mg

Alanine	2.0
Arginine.HCl	174.4
Asparagine.H2O	28.4
Aspartic acid	12.0
Cystine.2HCl	31.6
Glutamic acid	11.9
Glutamine	299.6
Glycine	12.8
Histidine.HCl.H2O	22.6
Hydroxyproline	9.5
Isoleucine	47.2
Leucine	49.6
Lysine.HCl	59.2
Methionine	14.8
Phenylalanine	22.3
Proline	16.6
Serine	25.5

Threonine	33.2
Tryptophan	5.5
Tyrosine	26.1
Valine	32.7
Glucose	3,423

Cystine is utilized in lieu of cysteine which is toxic to cells at high concentration.

The composition of medium BTC-28102 is:

Glucose	10.269 g/l
Amino Acids	15.628 g/l
Amino Acids (% d.w. of media ingredients)	41.1

Inoculum cells were adapted to Medium BTC-28101 following the protocol stated in Example 2 and inoculated at $2x10^5$/ml when starting the shaker batch, along with the control cells in 100 ml of DMEM/F12 medium. The effects on cell growth are shown in Fig. 4. Maximum concentration of Ig in the culture are summarized in Table XII.

Table XII: Maximum Ig Concentration in the cultures with BTC-28102 and Control DMEM/F12 Media

Cell Line	Max Ig Concentration (μg/ml) DMEM/F12	BTC-28102
2HG11	50	490
TBC3	84	1200

EXAMPLE 12

Preparation and Use of BTC-28103 to Culture CHO Cells

This invention illustrates use of the invention to culture mammalian cells that express natural or recombinant protein. BTC-28103 was prepared as in BTC-28101 but the buffer contents of HEPES and NaHCO were increased to 8330 mg/l and 2650 mg/l, respectively. As a result, osmolarity of the medium was increased to 360 mOsm/Kg. CHO (Chinese Hamster Ovary) cells were adapted to grow in suspension and cultured in 100 ml of BTC-28103 and the control IMDM in shaker flasks, both supplied with 10% FBS, thymidine and hypoxanthine. Growth of the cultures was followed daily by hemocytometer counting and presented in Fig. 5.

The media of the invention is useful to culture protein expressing cell lines in the various forms of available bioreactors. In particular, media of this invention may be used as the intracapillary medium in hollow fiber bioreactor culture of recombinant protein expressing CHO cells.

EXAMPLE 13

Table XIII indicates the composition of the commercially available media RPMI, D/F and eRDF and of the 28101 (Example 1) and 28102 (Example 11) media of the invention.

Table XIII

	RPMI	D/F	eRDF	28101	28102
Glucose mg/l	2.00	3.15	3.42	6.846	10.269
Amino acids mg/l	1.04	1.11	3.1	6.251	15.628
Amino acids (% d.w. of components)	5.6	6.6	16	24.8	41.1

The correlation of % amino acid content in medium with MAB production is presented in Fig. 6.

A novel cell culture media which improves protein production by cells of all types including mammalian cells which express recombinant protein vectors has been disclosed. The invention will substantially enhance the cost effectiveness of cell culture procedures generally including the production of monoclonal antibodies.

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(2) Chua, F., et al. (1994) Journal of Immunological Methods 167:109-119.

(3) Chua, F., et al. (1994) Journal of Biotechnology 37:265-275.

(4) Oh, S.K.W., et al. (1995) Biotechnology and Bioengineering 48:525-535.

(5) Oh, S.K.W., et al. (1996) "Flow Cytometric Studies of Osmotically Stressed and Sodium Butyrate-Treated Hybridoma Cells" in Flow Cytometry Applications in Cell Culture, Marcel Dekker, Inc., (Eds. M. Al-Rubeai and A.N. Emery) New York, Basel, Hong Kong, pp. 101-119.

WHAT WE CLAIM:

1. In a cell culture medium comprising an aqueous solution of amino acids and a carbohydrate energy source for cells cultured in said medium,

the improvement wherein said aqueous solution

(i) is substantially saturated with said amino acids at a temperature of 30° to 50°C;

(ii) has an osmolarity of 320 to 450 mOsm;

(iii) contains 5.50 to 20 grams per liter of amino acids and 5.50 to 15 grams per liter of said carbohydrate energy source

and wherein the dry weight of the amino acids in solution in said medium comprises at least 20% of the total dry weight of all solid components present in said medium.

2. The claim 1 cell culture medium in which said carbohydrate energy source is glucose.

3. The claim 1 cell culture medium in which said osmolarity of from 320 to 450 mOsm is provided at least in part by sodium salt.

4. The claim 1 or claim 2 cell culture medium in which said osmolarity of from 320 to 450 mOsm is provided at least in part by a sodium chloride osmolyte.

5. The claim 1 cell culture medium further comprising a suspension of undissolved amino acids in said aqueous solution substantially saturated with amino acids

wherein said substantial saturation is maintained as cell growth consumes amino acids in solution in said aqueous medium.

6. In an aqueous basal cell culture medium, the improvement which comprises providing in said basal medium

(i) a concentration of one or more amino acids reactive with cell transport system A to provide a total amino acid concentration of 5.50 to 20 grams per liter;

(ii) an osmolarity of from 320 to 420 mOsm said osmolarity being provided by a sodium salt osmolyte.

7. The claim 6 cell culture medium in which the osmolyte (ii) is sodium chloride.

8. The claim 6 cell culture medium in which said one or more amino acids reactive with cell transport system A are zwitterionic amino acids.

9. The claim 6 cell culture medium in which said one or more amino acids reactive with cell transport system A are selected from the group consisting of alanine, glycine, histidine, methionine, proline and serine.

10. The claim 6 or claim 7 or claim 8 cell culture medium in which said basal cell culture medium is RPMI, DMEM, Ham's F12, RDF or eRDF.

11. The cell culture medium 28101, 28102 or 28103.

12. The method which comprises culturing a cell in the culture medium of claim 1, claim 2, or claim 3, or claim 11.

13. A method which comprises:

(i) providing the cell culture medium of claim 1 or claim 2 or claim 3 or claim 11;

(ii) culturing a CHO cell containing a recombinant expression vector in the medium provided in step (i); and

(iii) recovering the recombinant protein expressed by said CHO cell.

14. The claim 13 method in which the CHO cell containing a recombinant expression vector is a dicistronic DHFR intron expression vector.

15. A method which comprises culturing a CHO cell in culture medium 28103.

16. The method of claim 13 wherein said cell cultured in said culture medium is a hybridoma.

17. The method which comprises culturing a mammalian cell in the culture medium of claim 1, claim 2 or claim 3 or claim 11.

18. The method which comprises culturing a mammalian cell having an expression vector for a recombinant protein in a culture medium of claim 1, or claim 2 or claim 3 or claim 11.

19. The method which comprises culturing a CHO cell or a BHK cell or a COS cell or a Namaliva cell having expression vector for a recombinant protein in a culture medium of claim 1, claim 2, claim 3 or claim 11.

20. The method which comprises culturing a cell in the culture medium of claim 6 or claim 7 or claim 8 or claim 9.

21. The method which comprises culturing a mammalian cell in the culture medium of claim 7 or claim 8 or claim 9.

22. The method which comprises culturing a mammalian cell having an expression vector for a recombinant protein in a culture medium of claim 7 or claim 8 or claim 9.

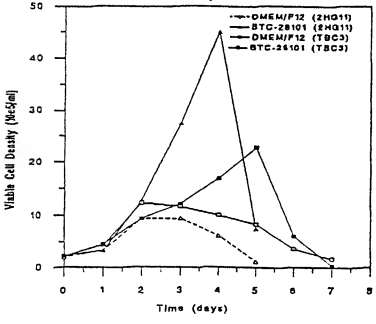
23. The method which comprises culturing a CHO cell or a BHK cell or a COS cell or a Namaliva cell having expression vector for a recombinant protein in a culture medium of claim 7 or claim 8 or claim 9.

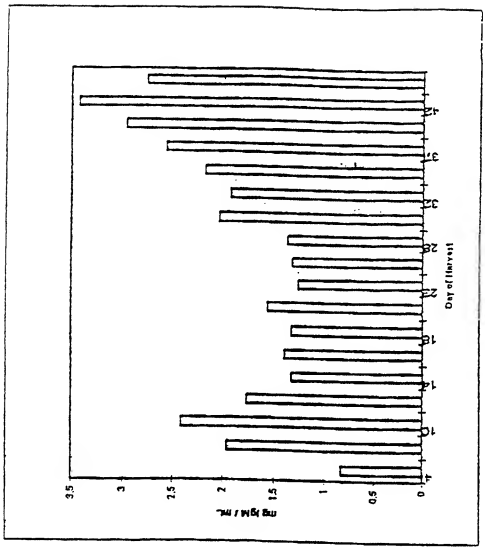
24. A dry mixture of cell culture medium components comprising amino acids, carbohydrates and vitamins,

wherein said amino acids component comprises at least 20% of the dry weight of said mixture.

25. The claim 24 mixture wherein said amino acids component comprises from 30% to 50% of the dry weight of said mixture.

Fig. 1 Growth of Hybridomas 2HG11 and TBC3 in BTC-28101 and Control DMEM/F12 Media

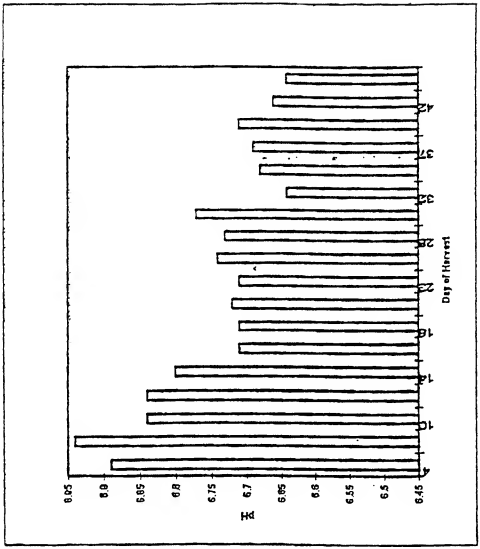




Day	IgM mg/mL
4	0.828
7	1.08
10	2.41
12	1.78
14	1.32
16	1.39
18	1.32
21	1.58
23	1.25
25	1.31
28	1.36
30	2.03
32	1.82
35	2.17
37	2.56
39	2.08
42	3.42
44	2.76

I = removal and replacement of I L media
II = removal and replacement of I L media

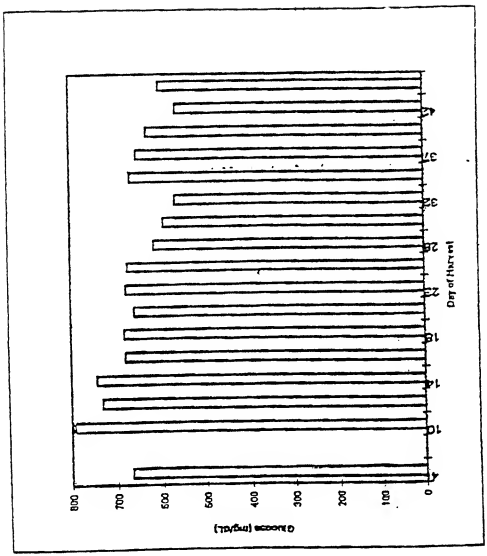
FIGURE 2A



Day	pH
4	6.89
7	6.84
10	6.84
12	6.84
14	6.8
16	6.71
18	6.71
21	6.72
23	6.71
25	6.74
28	6.73
30	6.77
32	6.84
35	6.68
37	6.69
39	6.71
42	6.66
44	6.64

I = removal and replacement of 1 L media
II = removal and replacement of 1 L media

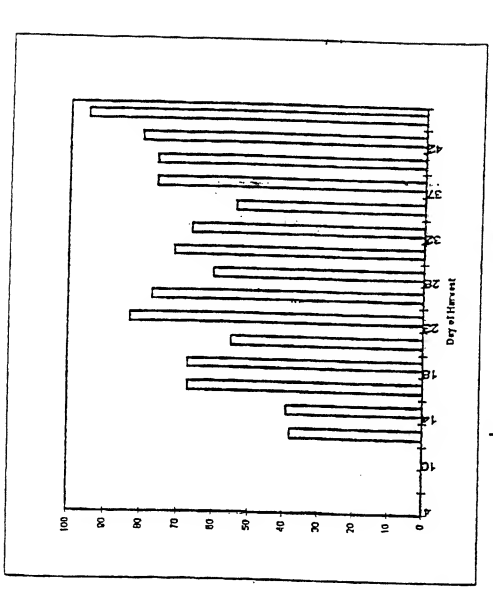
FIGURE 2B



Day	Glucose mg/dL
4	668
7	782
10	732
12	744
14	690
16	682
18	660
21	678
23	674
25	614
28	582
30	566
32	668
35	652
37	628
39	562
42	600
44	

I = removal and replacement of 1 L media
II = removal and replacement of 1 L media

FIGURE 2C



Day	% Cell Viability
4	ND
7	ND
10	ND
12	38
14	39
16	67
18	67
21	55
23	83
26	77
28	60
30	71
32	66
35	54
37	76
39	78
42	80
44	85

ND = not done
I = removal and replacement of 1 L media
II = removal and replacement of 1 L media

FIGURE 2D

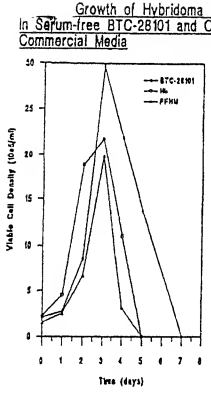


FIGURE 3A

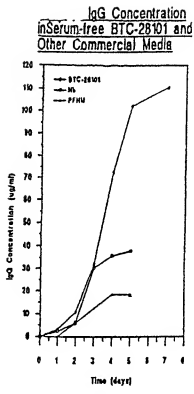


FIGURE 3B

Fig. 4 Growth of Hybridomas 2HG11 and TBC3 in BTC-28102 and Control DMEM/F12 Media

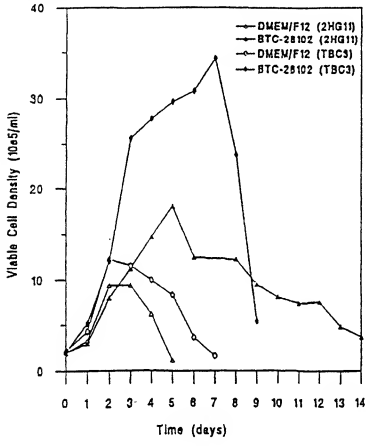


Fig. 5 Growth of CHO Cells In BTC-28103 and Control IMDM Media

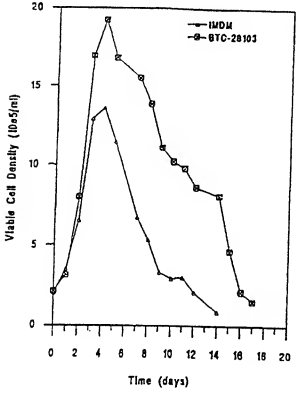
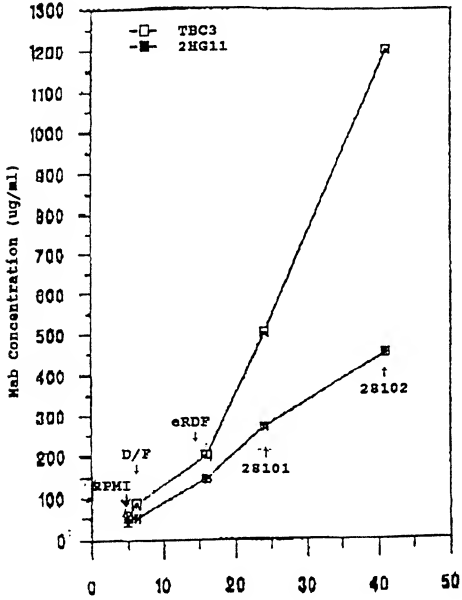


FIGURE 6

Correlation of % amino acid content in medium with Mab production



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/06800

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12N 5/00
US CL : 435/325
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, WPIDS, CAPLUS, MEDLINE
search terms: osmolarity, osmolality, amino acids, CHO

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 5,122,469 A (**MATHER** et al) 16 June 1992, abstract, column 6, lines 45-70, colum 7, lines 1-35, column 10, lines 60-70.	1-10, 12, 13, 17-23 --- 1-25
X	US 3,950,547 A (**LAMAR** et al) 13 April 1976, column 20, lines 47-60, column 7, line 21.	24,25
Y	US 4,724,206 A (**RUPP** et al) 09 February 1988, abstract, column 6, lines 15-70, column 7, lines 1-30.	1-25
Y	US 5,316,938 A (**KEEN** et al) 31 May 1994, abstract, column 3, lines 25-45, column8, linees 30-34.	1-23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
14 MAY 1998	30 JUL 1998
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/06800

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MURAKAMI, HIROKI. Serum-Free Medium Used for Cultivation of Hybridomas. In: Advances in Biotechnological Processes. Edited by A. Mizrahi. Alan R. Liss, New York: 1989, Vol 11, pages 107-141, especially page 111.	1-23
Y	CHUA et al. Hyper-Stimulation of Monoclonal Antibody Production by High Osmolarity Stress in eRDF Medium. Journal of Biotechnology. 1994, Vol.37, pages 265-275.	1-23